SEROLOGIC TECHNIQUES

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September 16, 1998

The goal of this discussion is to update you on advances in HIV diagnostics using standard serum based assays through four areas of discussion. The first is a brief overview of primary infection, focusing on an understanding of the sequential evolution of the window period dynamics of virus and antibody evolution. Next we will review the progressive improvements and some of the remaining problems with respect to screening and confirmatory antibody assays. I will next introduce a topic Dr. Janssen and I have worked on extensively, a less sensitive EIA strategy for detecting recent infections which allows one to both project incidence rates and identify recently infected people among seropositives for other public health and individual treatment applications. Finally, I will briefly address the potential role of adding direct virus detection assays to screening in both blood bank and general diagnostic contexts. For an extensively referenced review of the issues discussed here, the interested reader should see *Busch MP, Satten GA. Time Course of Viremia and Antibody Seroconversion Following Primary HIV Exposure. Amer J Med 1997; 102 (Suppl 5B): 117-126.*

In terms of primary infection, there are data from a number of sources that we have to piece together to get an overall understanding of the dynamics and time course of primary infection. Much work has been done in this area using serial donations from paid plasma donors who donated 2-3 times per week. Not infrequently, these donors seroconvert. Fortunately, the prior donations from these donors are available and are compiled by several companies into panels that allow us to study in great detail the dynamics of viremia. These resources have been very useful. Another source of information regarding primary infection comes from persons with known exposure dates, such as needle stick accident cases. In addition, a subset of people who present with primary syndrome have discreet exposures that allow us to look at the time period from exposure to evolution of markers or symptoms. Unfortunately, these cases typically have very few samples collected or available to go back to from time of exposure to presentation or seroconversion. Therefore, collectively, they are not as informative, although they do give us critical information regarding time from exposure to marker positivity.

Recipients of blood products from seroconverting donors is important from the blood bank perspective. This is the case when donors seroconvert and their prior donations have already been transfused. Large studies by Lyle Petersen and Glenn Satten at the CDC enabled us to understand the infectious duration prior to seroconversion by analyzing the relationship between inter-donation interval and transmission. Cohort studies often have samples available only at three to six month intervals, but these allow us to go back and test earlier bleeds once seroconversion has occurred. Analyzing large numbers of these samples gives us insight into the timing and duration of various marker positivity prior to seroconversion. These are important because they are the only source of cellular samples in which to look at the frequency of provirus detection prior to antibody formation.

Finally, animal model data are important. Although they have limitations in terms of direct extrapolation to humans, they allow us to elucidate certain principles that can be very important as we look at human data. Using an animal model of SIV where there is sub-mucosal inoculation of virus followed either by biopsy or sacrifice of animals demonstrates the concept of an eclipse phase. This is defined as the period following exposure to an infectious agent prior to the ability to detect any evidence of infectivity or marker positivity in the blood. This has been explained in studies by using these animal models. Specifically when these animals are biopsied or sacrificed over the first days to week following inoculation, the only evidence of infection is in the regional lymphoid tissue draining the inoculation site. Over the first few days, one detects infected Langerhans cells that have migrated to the draining lymph nodes. These draining lymph nodes become factories producing virus, prior to any evidence of systemic viremia either in the blood or other lymphoid tissue. Then the infection spreads, either via lymphatics or directly into blood. This demonstrates a transient phase following infection during which an individual may not have any markers of infection in their blood and may not be infectious. As we will see in the human data, there is strong evidence for the existence of such an eclipse phase.

The following discussion is data which have come from Larry Corey's Seattle group and which Schecter et al are beginning to expand upon. The data involve the time from exposure to when patients present with symptomatic seroconversion. There is a marked expansion in the networks acquiring patients into these early intervention and pathogenesis trials. Most of the current studies are recruiting patients who are found to have symptoms of a primary syndrome. Again, in a subset of these cases, the individuals relate a discrete sexual exposure that allows one to estimate the time from that sexual exposure to the presentation with symptoms. From a number of these studies, there is on average about a fourteen day period from the time of exposure to when patients present with symptomatic infection. Usually when patients present with the symptomatic infection, they are already near the peak antigenemic phase. Samples are usually unavailable to study the pre-antigenemic period in these patients.

CDC data concerning the healthcare worker study group in the United States involved fifty one cases of healthcare workers who became infected following a well-defined needle stick or other exposure. When a survival curve is created with these data approximately half of the individuals were estimated to have tested antibody positive by the tests used at the time at about forty days following the exposure event. The tests were quite variable as these data were accrued over approximately ten years. Some of the tests were early generation assays that had less sensitivity than the current assays. It is quite possible then that the forty day average time from exposure to seroconversion may have actually been to twenty or thirty days. Nonetheless, there is an average twenty to forty day time period from exposure to antibody seroconversion based on this analysis.

Importantly, a small number of outlier cases took up to six months to seroconvert. These outlier cases have been studied rigorously and have been genetically sequenced and compared to the source of the needle stick to verify that these were acquisitions attributable to the needle stick exposure. In a small number of these cases, where samples happened to be available from earlier time points, those samples have been tested and have been found to be consistently RNA and p24 antigen negative at the earlier time points. Only in the bleed immediately prior to seroconversion do these individuals become viremic. These data suggest that these delayed cases of seroconversion are probably attributable to delayed dissemination, probably a prolonged

eclipse phase, not a prolonged phase of viremia that could be detectable or infectious.

Panels of serial units from plasma donors were identified based on their seroconversion as detected by standard antibody assays. Fortunately, there were large volumes of frozen plasma components that we could go back to prior to pooling, and then test the earlier bleeds. We have done work on well over fifty of these types of panels and the data are extraordinarily consistent from panel to panel in terms of the dynamics of the RNA ramp up, decline and stabilization, p24 antigen detection, and the serial evolution of seroconversion to antibodies detected either with the third generation tests or the less sensitive IgG direct antibody assays.

By analyzing large numbers of these panels, we have been able to estimate and define the stages of evolution of viral markers. The data can be summarized by two parameters: stage duration and viral load distribution. The stages that we can define from these data are a brief period when only RNA is detectable and p24 antigen tests and antibody are negative (Stage 1). That stage is estimated to last about three and a half days. This is followed by a period where the levels of virus increase such that antigen is also detectable, but in the absence of any detectable antibody by third generation assays. (Stage 2)This stage is estimated to last about five days. Then seroconversion is detectable by the very sensitive IgM detection EIA assays, but the Western blot is negative for about three days (Stage 3). The blot then goes through a phase, lasting about five days, where a few bands appear but the result is indeterminate (Stage 4). Then there is a stage that lasts about thirty-five days when the blot is technically positive by current two band criteria, but would not have been positive by the earlier three band criteria (Stage 5). In particular, the p31 band does not mature in the blot until over a month after the blot is positive by two band criteria. We will revisit this issue later.

These data demonstrate the progressive stages of seroconversion. During Stage 1, the RNA load on a population basis proved to be quite low, ranging from the lower limit of the sensitivity of the assay of approximately 400 copies to about 10,000 during the RNA only stage. Once the RNA levels reach 10,000 copies, p24 antigen is consistently detected (Stage 2), and then as the antibody matures, we see the viral load drop (Stages 3-5).

In a number of the panels, we have been able to do doubling time analysis based on two or three RNA data points on the up slope of viremia prior to any antibody detection. In those panels we calculated an average doubling time of about 0.9 days, with a range of 0.4-1.4 days. This showed a rather consistent ramp up rate of viremia. With the panels that we have developed, we can retrospectively estimate when in each panel the viral load would have had a theoretical single copy per milliliter. If we then plotted all of these panels on a single time line, based on this extrapolated viral load doubling time, you would see quite a consistent ramp up of viremia in primary infection, prior to detectable RNA, with a very brief period of only five to ten days during which the viral load is quickly increasing. Therefore, there is a very brief predicted time period during which there is a sub-detectable level of viral load.

Combining the needle stick exposure and plasma donor panel data leaves us with the concept of a post-exposure proviremic eclipse phase, which is thought to be generally quite brief, perhaps on average about ten days, but which can occasionally be longer, up to six months. It is followed by the progressive stages of detectable viremia which are almost undoubtedly infectious, from the blood bank perspective. For us, one of the critical questions is, are these pre-viremic samples infectious? To address that, a number of studies are underway. One such study involved a chimpanzee with HIV. The chimpanzee was sampled at weekly intervals, became viremic at

week five, and seroconverted downstream. Each of the weekly samplings were processed and frozen as cells and plasma. We went back to two weeks prior to any detectable viremia and inoculated that blood sample into a second chimp. That chimp was then monitored for three months. It did not seroconvert and there was no evidence of infection. We then inoculated the chimp with the sample from week four, which was one week prior to detectable viremia. Again, no transmission occurred following inoculation. Finally, with the sample from week five, when the animal became viremic, we observed secondary transmission with the classical evolution of markers. This suggests that the pre-detectable viremic phase is also non- infectious. Further studies along this line are underway. The important question that we are left with is, will nucleic acid screening completely eliminate the potentially infectious window phase?

Looking historically at antibody tests, we have gone from early first generation assays, which were based on crude viral lysates and indirect antibody sandwich formats, to second generation assays, which had purified HIV 1 and HIV 2 antigens, or recombinant or peptide antigens, but continued to use an indirect EIA format. The switch to third generation assays was very important in enhancing sensitivity.

In the third generation assays, termed sandwich antigen tests, instead of the human antibody binding to the solid phase antigen followed by detection of the human antibody using an enzyme conjugated to anti-human antibody, the third generation assays have enzyme conjugated to the antigen again. In essence, the human antibody is sandwiched between antigen bound to the solid phase and antigen bound to the detection conjugate. The advantage plays out with IgM because it has multiple antibody-antigen binding sites allowing it to bind down large numbers of the antigen conjugate molecules for each IgM molecule detected. As a consequence, one sees enhanced signal very early following seroconversion. This improved sensitivity in the window period has been demonstrated by using a number of seroconversion panels. The data were compiled from a large number of panels based on the first detection of p24 antigen. The early assays were first licensed in 1985, and left us with a fairly long (approximately 20 days) antigen positive/antibody negative window period. The second generation tests partially closed that window (to approximately 10 days). The third generation tests almost completely closed the antigen positive/antibody negative window period.

Currently being developed and beginning to be implemented in Europe are fourth generation assays which combine antigen and antibody detection into a single assay. This assay combines the configuration of a monoclonal p24 antigen capture assay with envelope antigen-based antibody detection using a third generation format assays. These assays use chemiluminescent, detection which has enhanced performance in terms of the sensitivity of antibody detection. A particular example by Roche actually uses four different p24 monoclonals that allow detection of p24 antigen from both group M and group O HIV 1, recognizes IgG and IgM by using the third generation format for gp41, and now has specific subtype O and HIV 2 antigens represented. These fourth generation assays are designed to overcome the limitations of subtype detection as well as to couple the detection of antigen with antibody thereby allowing closure of the window period. The data indicate that the window period reduction from the first and second generation tests was reduced by about six days when going to the third generation tests. These combination antigen/antibody tests have now further closed the window comparably. You do not need to add antigen as a discrete assay. You are able to run a single screening test and benefit from the sensitivity of both antigen and antibody detection.

Regarding subtypes, HIV 2 became an obvious problem in the mid-1980s. Blood banks switched uniformly in the early 1990s to HIV 1/2 combination tests. Currently, over one hundred HIV 2 infections have been detected in the United States. Despite that, it is interesting that most public health sector labs in the U.S. still use HIV 1 tests, primarily because of the marked cost differential between the second generation HIV 1 assay and the third generation HIV 1/2 combination assays and the complexity of confirmatory tests. If you screen with a combination test, you have to sort out which virus might be present. An example is the HIV 1 group O, a problem which came to light in the mid 1990s and is still not resolved in terms of testing. The time line within FDA is dramatic in terms of getting tests licensed. Interestingly, the FDA has mandated manufacturers incorporate group O not only into the screening antibody test but into nucleic acid screening. However, they do not mandate detection of HIV 2. To date there are only two or three group O infections in the United States relative to hundreds of HIV 2 infections. Yet HIV group O screening seems to be the focus. Perhaps the political visibility of these rare variants has led FDA to focus the manufacturers on detection of this rare variance, while ignoring what I think is a much more substantial and increasing representation of HIV 2.

The non B subtypes are also a screening challenge. One recent paper published in AIDS focused on the relative sensitivity to seroconversion of different screening tests for B and non B subtypes. The study identified B and non B seroconverters from an African population who were detected during the antigenemic phase by the combined use of p24 antigen and the standard antibody assays. The study then examined the improvement in seroconversion detection by the third generation format assays among the two study groups. Focusing on Abbott's testing evolution with respect to the type B infections, the sensitivity for these early antigen positive samples went from forty to ninety percent by moving to the third generation antigen sandwich format, which, importantly, is based on group B antigens. In contrast, there was no improvement in the detection rate of antigen positive samples for non B subtypes. As non B subtypes begin to enter a population, we need to be concerned about improving the window period sensitivity for non B subtypes, because the current format assays are not designed to address this issue. Non B subtypes are beginning to be detected in the United States at an increasing rate. In several recent studies from the northeastern region where large numbers of immigrants and persons from endemic countries are screened, significant numbers of non B subtype infections have been identified.

This is also becoming an issue in the donor setting. A CDC surveillance study in which we subtyped approximately 400 HIV infected donors by heteroduplex mobility and identified two A and one C infection. These were sequence confirmed. One of the A cases was a United States transmission. This indicates a secondary spread of subtype A in the United States. Clearly, these non B viral strains are spreading in the United States, not only through immigration, but via secondary transmissions.

In terms of confirmatory testing, the bulk of the work is still done with viral lysate Western blots. These have performed well, but they have some substantial problems. One that I will touch on is, the sensitivity of screening tests have improved to such an extent that the supplemental tests failed to detect these early samples as positive. That leaves us with a concern over EIA reactive, blot negative or blot indeterminate samples. Could they represent real infections? Another problem is the high indeterminate rates. Finally, we have recently described false positivity of the confirmatory Western blots.

Immunofluorescence assays perform quite well in terms of sensitivity. However, very few places have implemented them because of subjective

interpretation issues. Recombinant antigen-based immunoblot assays have improved sensitivity and specificity, and build into the single confirmatory test viral type discrimination. Unfortunately, these tests are not licensed in the United States, and the FDA barrier to approval of these tests is very difficult to overcome.

Using second generation antibody assays, data from seroconversion panels have shown that seroconversion was not seen until day 21 post RNA detection. The third generation assay closed the window to day seven. Importantly, the Western blot was actually negative early following EIA seroconversion, and then went through early indeterminate stages. Although in the blood bank setting, donors with indeterminate western blots are virtually never infected, in public health settings, it is possible that a true seroconverter could be detected as Western blot indeterminate. Another problem with the Western blots is the misinterpretation of false positive blots. Some samples have shown high levels of gag related reactivity in the vicinity of gp41. These can and have been misclassified as gp41, and can lead to a false positive interpretation, based on p24, gp41 bands. There is litigation over cases like this. A more common problem that we have detected frequently in blood banks (JAMA 13 Sept., 1998) is double envelope false positive results, with gp41, gp120, gp160. Data from our laboratory have illustrated that serial bleeds over a period of years showed that donors who have had these two band positive results (these are called positive with the current criteria, which no longer require p31) are non infected. They do not evolve serologically and are negative for HIV RNA. In studies done in blood banks, about half of the blood donors who have patterns that are technically positive by envelope or p24 envelope criteria but lack p31 are not infected, based on PCR studies and follow up.

We detect these non-infected but technically positive donors at a rate of about of one in one hundred thousand screened individuals. For quite a while these donors were told they were infected, but in fact, they are not. False positive Western blots are a real problem in the blood bank setting. You can predict whether samples that meet two band criteria but lack p31 are likely to be real or not based on the additional bands. Basically, the more bands the samples have in addition to the double envelope pattern, the more likely it is that these samples will be viremic. Again, samples that are technically positive by the current two band criteria, but lack p31 should alert one that those samples may be false positives. The probability that they are false positives is related to the underlying incidence, or the probability that one would have a true seroconverter in that early seroconversion phase. The JAMA paper illustrates that in the low incidence blood bank setting where we have an incidence of only four per hundred thousand, only very rarely would a donor be estimated to be truly infected during that evolving Western blot phase. Therefore the frequency of false positivity is approximately as common as early infection with this evolving Western blot pattern. As you go to higher incidence populations, the contribution of this false positivity to misinterpretation is much lower. In high incidence settings, 90% of samples from people who meet the two band positive criteria would be infected. Still, this is an area to be alert to and concerned about.

The future of serology confirmatory testing in other countries, and hopefully in the United States, should move to recombinant antigen based assays. The Chiron-RIBA test is an example of such an assay which has most of the immunodominant epitopes for HIV 1 and HIV 2, while incorporating good group O sensitivity, and allowing for discrimination among the groups and subtypes.

The last issue I am going to address is development of a less sensitive or “detoned” HIV antibody assay. In a recent JAMA paper, we

reported on a test which is a modified Abbott lysate EIA where we have reduced the concentration of the sample by increasing its dilution and also reduced incubations and set a higher cut off. In contrast to the standard assays which peak and plateau within days of antibody detection, seroconversion evolves more slowly over time with a less sensitive assay. Over the first 120 days or so following standard seroconversion, virtually everyone who was recently infected will be classified by the less sensitive assay as a recent seroconverter. Once you are beyond 150 days from seroconversion by standard methods, virtually everyone is detected as reactive on the less sensitive assay and therefore considered to be of long standing infection.

We have looked at a number of populations with varying incidence ranging from one to two percent, as is seen in high risk populations, to a blood donor population where the incidence is one to two per 100,000. The results validated the approach of using a de-tuned assay to detect recent infections to project incidence estimates that are comparable with the observed incidence rates in these populations.

We are applying this method to large populations using cross sectional sample sets. Large studies of first time blood donors where we have been able to project incidence rates in the range of seven per 100,000 have been quite stable over time. Now we can look at the incidence in blood donors broken up by demographics and risk factors. Other studies involve prisoners and anonymous testing clinic settings, settings where we traditionally see incidence rates of two percent. We are then able to look at risk factors or other correlates of incidence. In studies of early HIV treatment we are now enrolling people based on this less sensitive EIA. The availability of this test has increased the ability to identify people with early infection by >50% for enrollment into treatment trials.

Another interesting observation regarding the less sensitive assay was seen in patients recruited into early intervention trials. In concert with a clearance of viral load following early, highly active antiretroviral drug treatment, these individuals stop seroconverting and in fact serorevert on less sensitive format tests. Conversely, we observed a rebound effect in patients who stopped treatment. There was a viral load increase followed by evolution of seroconversion. This suggests that a simple format, less sensitive antibody test, could have value in the context of monitoring treatment efficacy and compliance to drug therapy.

So, where are we going from here? There are a large number of epidemiologic studies in progress trying to identify high incidence populations, so that we can target resources and counseling, and focus contact tracing resources on the high incidence subsets. We are focused in the blood bank setting on identifying the opposite, i.e., the lowest incidence group to target our donation recruitment efforts. Studies are ongoing in HIVNET to identify high incidence populations for vaccine trials and monitoring vaccine efficacy. This method also allows for identification of samples from recently infected people to focus molecular surveillance of subtypes and drug resistance or envelope diversity analysis on the recently transmitted strains which are the leading edge of the epidemic. On an individual basis, identifying recently infected people among the positives allows for early referral treatment of these clients. By focused contact tracing on recently infected people, where the yield is predicted to be much higher, we will hopefully be better able to identify and interdict transmission networks.

I want to close by briefly addressing direct virus detection issues. Given the very short durations of the viremic stages prior to seroconversion, the yield of adding antigen or RNA is very low. However, in the blood bank setting, we were mandated several years ago to add p24 antigen

testing. Within the next year, we will be mandated to add HIV and HCV RNA screening. Based on pilot studies yield has been very low with numerous complications. Despite this, these assays, particularly the very sensitive qualitative RNA tests, are maturing. They must be implemented in blood banks via mandate. However, I would just suggest that studies be performed to look at the potential utility of these assays in diagnostic screening settings. In higher incidence populations, screening for HIV RNA at a cost of $5.00 or $10.00 may prove to have utility in terms of detection of people with early infection who are potential candidates for early treatment. There is also the possibility that these are the most infectious individuals, and if so, expeditious detection and early treatment could have broad public health implications.